THE USE OF *UNIO TUMIDUS* FOR DETECTION OF WATER POLLUTION

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Abstract

The present research tries to shed some light on the field of biomonitoring. We recorded the behaviour of mussels, *Unio tumidus,* and the way they react depending on the type of substances that are in the water. The final aim is to build an early warning system to reduce the time of human response against sudden water pollution. A total of 40 mussels were studied at the laboratory of Department of Ecology and Environmental Protection in Poznan University of Life Science, from March to June, 2016. The present study collects the reactions of these mussels to different substances at different concentrations, namely: 0'2 mg L^{-1} and 1 mg L^{-1} of Fe^{+3}, 10 mg L^{-1} and 25 mg L^{-1} of NH_4NO_3 and 10 mg L^{-1} of $NO^{-}_{3(aq)}$. Different reactions to different substances and concentrations were observed. However, since mussels behaved very similarly to the same substance, we could recognize certain patterns of behavior to identify each substance. Another observation is whenever the mussels react slowly to any change to the environment (i.e. when a substance is added), it indicates that mussels are trying to adapt to the new conditions. On the other hand, if the mussels react fast, then there is no possibility of resist and to survive to the new conditions. The reaction rate of the mussels increased in the following order: 0'2 mg L^{-1} Fe^{+3} >>> 1 mg L^{-1} Fe^{+3} >> 10 mg L^{-1} de NO_3^- > 10 mg L^{-1} NH_4NO_3 > 25 mg L^{-1} NH_4NO_3. It means that the mussels are more resistant to Fe: this may be due to their ability to accumulate heavy metals. Furthermore, we observed some variations in behavior that were attributed to 'individual personalities': there were individuals that behaved differently to what was expected or commonly established, which could be related to a state of poor health, old age, different sizes and sex. Further research is needed to fully understand mussels' behavior when they are exposed to different substances (as analysed in the current study) as well as to other substances that may be harmful for human health and can be found in polluted water. In addition, further work needs to be undertaken to standardize mussels' biomonitoring as an early warning system.

Keywords *Unio tumidus* · mussel · biomonitoring · behavior

Resum

El en present treball s'ha intentat obrir pas en el camp del biomonitoreig. Es va registrar el comportament dels musclos, *Unio tumidus*, i la forma en què reaccionen segons el tipus de substàncies que es troben en l'aigua. L'objectiu final és la construcció d'un sistema d'alerta primerenca per reduir el temps de resposta humana contra una contaminació sobtada en l'aigua. Un total de 40 musclos van ser estudiats al laboratori del Departament d'Ecologia i Protecció Ambiental a la Universitat de Poznan de Ciències de la Vida, de març a juny de 2016. Les substàncies i les concentracións utilitzades van ser les següents: 0 '2 mg L^{-1}, 1 mg L^{-1} de Fe^{+3}, 10 mg L^{-1} i 25 mg L^{-1} de NH_4NO_3 i 10 mg L^{-1} de $NO^{-}_{3(aq)}$. Es van observar diferents reaccions amb substàncies y concentracions diferents. No obstant, els vuit musclos d'una mateixa prova, es van comportar de manera molt similar davant la mateixa substància, d'aquesta manera podríem reconèixer certs patrons de comportament per a identificar cada substància. Una altra observació és, sempre que els musclos reaccionen lentament a qualsevol canvi en el medi ambient (és a dir, quan s'afegeix una substància), indica que els musclos estan tractant d'adaptar-se a les noves condicions. D'altra banda, si els musclos reaccionen ràpid, llavors significa que no hi ha possibilitat de resistir i sobreviure a les noves condicions. La velocitat de reacció dels musclos va en augment en el següent ordre: 0'2 mg L^{-1} Fe^{+3}>>> 1 mg L^{-1} Fe^{+3} >> 10 mg L^{-1} de NO_3^- > 10 mg L^{-1} NH_4NO_3 > 25 mg L^{-1} NH_4NO_3. Això vol dir que els musclos són més resistents a Fe: això pot ser causa de la seva capacitat d'acumular metalls pesants. D'altra banda, s'aprecien algunes variacions en el comportament que es van atribuir a "personalitats individuals": hi va haver individus que es comportaven de manera diferent al que s'esperava o comunament establert, que podrien estar relacionats amb un estat de mala salut, l'edat, tamany i sexe. Es necessita més investigació per entendre completament el comportament de musclos quan estan exposats a diferents substàncies (com s'analitza en el present estudi), així com d'altres substàncies que poden ser perjudicials per a la salut humana i poden ser trobats en l'aigua contaminada. A més, un treball addicional és necessari dur a terme per estandarditzar la biomonitorització de musclos com un sistema d'alerta primerenca.

Paraules clau Unio tumidus · musclo · biomonitorització · comportament

Resumen

El en presente trabajo se intent abrir paso en el campo del biomonitoreo. Se registró el comportamiento de los mejillones, *Unio tumidus*, y la forma en que reaccionan según el tipo de sustancias que se encuentran en el agua. El objetivo final es la construcción de un sistema de alerta temprana para reducir el tiempo de respuesta humana contra una contaminación repentina en el agua. Un total de 40 mejillones fueron estudiados en el laboratorio del Departamento de Ecología y Protección Ambiental en la Universidad de Poznan de Ciencias de la Vida, de marzo a junio de 2016. Las sustancias y las concentraciones utilizadas fueron las siguinetes: 0'2 mg L^{-1}, 1 mg L^{-1} of Fe^{+3}, 10 mg L^{-1} y 25 mg L^{-1} of NH_4NO_3 y 10 mg L^{-1} of $NO^{-}_{3(aq)}$. Se observaron diferentes reacciones con diferentes sustancias y diferentes concentraciones. Sin embargo, los ocho mejillones de una misma prueba, se comportaron de manera muy similar ante la misma sustancia, de esta manera, podríamos reconocer ciertos patrones de comportamiento para identificar cada sustancia. Otra observación es, siempre que los mejillones reaccionan lentamente a cualquier cambio en el medio ambiente (es decir, cuando se añade una sustancia), indica que los mejillones están tratando de adaptarse a las nuevas condiciones. Por otro lado, si los mejillones reaccionan rápido, entonces significa que no hay posibilidad de resistir y sobrevivir a las nuevas condiciones. La velocidad de reacción de los mejillones va en aumento en el siguiente orden: 0'2 mg L^{-1} Fe^{+3} >>> 1 mg L^{-1} Fe^{+3} >> 10 mg L^{-1} de NO_3^- > 10 mg L^{-1} NH_4NO_3 > 25 mg L^{-1} NH_4NO_3. Esto significa que los mejillones son más resistentes a Fe: esto puede ser debido a su capacidad de acumular metales pesados. Por otra parte, se aprecian algunas variaciones en el comportamiento que se atribuyeron a "personalidades individuales": hubo individuos que se comportaban de manera diferente a lo que se esperaba o comúnmente establecido, que podrían estar relacionados con un estado de mala salud, la edad, el tamaño y sexo. Se necesita más investigación para entender completamente el comportamiento de los mejillones cuando están expuestos a diferentes sustancias (como se analiza en el presente estudio), así como de otras sustancias que pueden ser perjudiciales para la salud humana y pueden ser encontrados en el agua contaminada. Además, un trabajo adicional es necesario llevar a cabo para estandarizar la biomonitorización de mejillones como un sistema de alerta temprana.

Palabras clave Unio tumidus · mejillón · biomonitorización · comportamient

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Summary

Abstract **1**

Resum **2**

Resumen **3**

Acknoledgements **4**

List of Figures **7**

List of Tables **10**

1. Introduction **11**

1.1 The use of biological indicators in monitoring *11*

1.2 The impact of water pollutions on aquatic organisms *12*

1.3 The reaction of mussels to water contamination *13*

1.4 Mussels characteristics (morphological and physiological) which determine their sensitivity to pollutions *14*

2. Aim **16**

2.1 Testing of the suitability of Unio tumidus for the monitoring of water quality *16*

2.2 Analysis of the response of mussels to the presence of nitrate, ammonium nitrate and iron in water *16*

3. Methodology **18**

3.1 Description of the biomonitoring system *18*

3.1.1 Type of tank and materials 19

3.1.2 Preparing the mussels to research (quarantine period and its significance, the age of mussels, the number of individuals in the experiment) 21

3.1.3 Calibration system 23

3.1.4 System errors 25

3.2 Description of the analysed substances 29

3.2.1 Dosing of substances 29

3.2.2 Analysis of the results 29

4. RESULTS 32

4.1 Analysis of the response to the added substances 32

4.2 Analysis of the variation in response to various substances 34

4.3 Analysis of the variation in response of individual mussels 38

5. Discussion 42

6. Conclusion 43

7. Bibliography 44

8. Appendix 46

List of Figures

Figure 1. This simplified illustration shows the arrangement of the soft tissue body parts of a freshwater 14

Figure 2. Both pictures of Unio Tumidus in natural state 15

Figure 3. Measuring device set-up. water intake (ujęcie wody); water treatment station (stacja uzdatniania wody); inlet water (odbiorcy wody); computer pc (komputer pc); controller (sterownik); tank (zbiornik) Source: http://duszpasterstwo-wodkan-ochrsrod.pl/2012/07/18/robota-dla-mieczakow/ 18

Figure 4. Picture taken in the laboratory of the faculty in which we can identify the final set-up used for the present research 20

Figure 5. Pump impulsion. The substances are dosed by the pump that we installed in the system. This solution allows to run a non- invasive introduction of testing substances. 20

Figure 6. Photography of the quarantine aquarium, there are free mussels and mussels stuck onto the socle to be installed later in the research system 21

Figure 7. Preparing the socle still wet with the silicon to steck the mussel. 22

Figure 8. Visual explanation of the mussel placement above the support. The two images framed in red are misplacement and picture framed in green it is correctly oriented on the support. 22

Figure 9. Mussels already installed and ready for biomonitoring. 23

Figure 10. The taskbar of the main window of the system. 25

Figure 11. Bad calibration. 26

Figure 12. Good calibration 26

Figure 13. Result of good calibration 27

Figure 14. Good water quality 27

Figure 15. Bad water quality 28

Figure 16. Bad water temperature 28

Figure 17. This graphic shows the mussles' behavior's change collecting all datas of more than one day of ammonium nitrate with 10mg l^{-1} (A) and 25mg l^{-1} (B) 30

Figure 18. Control behavior of the eight mussles during 24 hours 32

Figure 19. Behavior of the mussels with 0,2 mg/l of Fe^{+3} during 24 hours. 33

Figure 20. Behavior of the mussels with 1 mg/l of Fe^{+3} during 24 hours. 33

Figure 21. Behavior of the mussels with 10 mg/l of NH_4NO_3 during three hours. 33

Figure 22. Behavior of the mussels with 25 mg/l of NH_4NO_3 during three hours 34

Figure 23. Behavior of the mussels with 10 mg/l of $NO^-_{3(aq)}$ during three hours 34

Figure 24. Graphical representation of the average percentage of opening every 15 minutes from the eight mussels without any substance 35

Figure 25. Graphical representation of the average percentage of opening every 15 minutes from the eight mussels with 0,2 mg/l of Fe^{+3} 35

Figure 26. Graphical representation of the average percentage of opening every 15 minutes from the eight mussels with 1 mg/l of Fe^{+3} 36

Figure 27. Graphical representation of the average percentage of opening every 15 minutes from the eight mussels with 10 mg/l of NH_4NO_3 36

Figure 28. Graphical representation of the average percentage of opening every 15 minutes from the eight mussels with 10 mg/l of NH_4NO_3 37

Figure 29. Graphical representation of the average percentage of opening every 15 minutes from the eight mussels with 10 mg/l of $NO^-_{3(aq)}$ 37

Figure 30. Representation in bars percentage of time spent in each position along with the standard deviation of the eight mussels without any substance 38

Figure 31. Representation in bars percentage of time spent in each position along with the standard deviation of the eight mussels with 0,2 mg/l of Fe^{+3}. 39

Figure 32. Representation in bars percentage of time spent in each position along with the standard deviation of the eight mussels with 1 mg/l of Fe^{+3} 39

Figure 33. Representation in bars percentage of time spent in each position along with the standard deviation of the eight mussels with 10 mg/l of NH_4NO_3 40

Figure 34. Representation in bars percentage of time spent in each position along with the standard deviation of the eight mussels with 25 mg/l of NH_4NO_3 40

Figure 35. Representation in bars percentage of time spent in each position along with the standard deviation of the eight mussels with 10 mg/l of $NO^-_{3(aq)}$ 41

List of Tables

Table 1. Experimental routine during seven days for each substance. 29

Table 2. Descriptive statistic of control' behavior of the eight mussels 46

Table 3. Descriptive statistic of the mussels' behavior with 0,2 mg l^{-1} Fe^{+3} in the water 46

Table 4. Descriptive statistic of the mussels' behavior with 1 mg l^{-1} Fe^{+3} in the water 47

Table 5. Descriptive statistic of the mussels' behavior with 10 mg l^{-1} nh_4no_3 in the water 47

Table 6. Descriptive statistic of the mussels' behavior with 25 mg l^{-1} nh_4no_3 in the water 48

Table 7. Descriptive statistic of the mussels' behavior with 10 mg l^{-1} $NO^-_{3(aq)}$ in the water 48

1. Introduction

The 21st century represents an era in which everything is being digitized; as a consequence, technology is increasingly present in more fields, including biomonitoring systems applied to detect levels of pollution that threaten human and animal life. For many years, scientists have been working with bioindicators in order to receive information about ecological characteristics of the environment or the impact of certain practices, as a way to assess the environment.

The present study was designed to biomonitoring the behaviour of *Unio tumidus*, commonly known as the swollen river mussel, controlled by a sensor which measures the opening shells, recording how these mussels react depending on the type of substances that are in the water. We have registered the reaction of the mussels to iron (Fe), ammonium nitrate (NH_4NO_3) and nitrate ($NO^-_{3(aq)}$) in order to build an early warning system to reduce the time of human response against sudden water pollution.

1.1 The use of biological indicators in monitoring

Nowadays, there are many studies that examine different types of indicators to determine changes in water quality (Kramer *et al.,* 1989). However, an increasing importance is being given to biological sensors, since the information these type of sensors provide is invaluable: by understanding how and why changes in mussels' normal state in the aquatic environment take place, we can detect water pollution.

Normally, physicochemical techniques are used to carry out water status monitoring. There are studies that monitor *Sinanodonta woodiana,* members of the mussel family, tracking DNA damage (Stoimir Kolarevi´c *et al*., 2013); as well as with *Pelophylax ridibundus,* that belongs to the family of true frogs, also known as the marsh frog, to measure metal accumulation (Slavica S. Borkovi´c-Miti´c *et al.,* 2016); there are also some studies based on the change in rheotaxis, respiration, gill activity or electrical field alterations in fish (Juhnke & Besch, 1971; Poels, 1977; Grubber & Diamond, 1998) among others.

Currently, the response of different biosensors is being used as an indicator to detect pollution in the aquatic environment (Cairns, 1979; Bayne et al, 1985). We found that bivalves offer many

possibilities with respect to continuous and automatic detection: they provide a reliable and fast response making data easy to be interpreted, and due to their small size, eating habits, maintenance and behaviour, they are very easy to work with. Therefore, bivalves, as an optimal functioning organism, could be considered as a suitable biological warning system.

Different applications for the use of the valve closure response can be developed, especially for environments where higher degree of toxicological conditions are to be expected. Since both freshwater mussels and marine mussels seem to react comparably, the proposed biosensor can be applied anywhere in the aquatic environment. Possible applications include:

- Effluent monitoring (discharge pipes);
- General water quality monitoring (rivers, coastal environments);
- Monitoring of water inlets (drinking water, a water sampler for chemical testing);
- Toxicity testing;
- Physiological and behavioural studies.

1.2 The impact of water pollutions on aquatic organisms

We must bear in mind that water represents three quarters of our planet and plays a very important role in the environment. It is distributed in different ways along the surface of the land and in rivers, lakes, swamps, glaciers, groundwater and wetlands. This means that water could easily distribute any type of substance and it can affect the environment.

Current scientific evidence on the presence of heavy metals in the soil, water and atmosphere supports and recommends a continuous monitoring of water quality to prevent ecological problems that could affect food properties and human health.

The consequences of contaminated water can result in a damaged ecosystem; once the biodiversity is modified, it may consequently lead to the disappearance of some organisms. Water pollution also affects the eutrophic chain, being harmful to humans, animals and aquatic life (e.g.: pollutants such as lead and cadmium are eaten by tiny animals; Later on, these animals are eaten by fish and shellfish, and the food chain continues to be disrupted at all higher levels). As I have previously stated, a continual monitoring of the presence of different pollutants and their impact on living organisms is essential to avoid an environmental catastrophe.

1.3 The reaction of mussels to water contamination

The reaction of mussels when the water conditions are not suitable for them is to close the shells immediately. Mussels are continually exposed to the aquatic environment; they are able to filter (mainly due to food intake) large quantities of water: between 15 and 45 liters per day (Strayer, 2008). Thus, the concentration of pollutants in molluscs can serve as an indicator of the level of pollution. Mussels have been widely adopted in chemical monitoring and surveillance programmes. Their ability to accumulate toxicants makes them suitable for the characterization of specific ecosystems.

In the passive version of chemical biomonitoring, local pollutants are sampled for chemical analysis, whereas the active version involves the exposure of organisms trans-located from reference sites/their original place. This disadvantage of this bioaccumulation is that the equilibrium concentration is usually obtained only after several weeks of exposure, making them unsuitable as an early warning system.

In contrast to the former method, physiological and behavioural reactions occur usually faster, making mussels potentially suitable as a fast response in continuous biological monitoring.

The valve movement of both freshwater and marine mussels has attracted much attention. The method is based on the fact that most mussels have their shells open for respiration and feeding most of the time. It has been shown that they close their shells whenever they are under stress for an extended period of time. Fluctuations in shells openness may be connected to the quantity of pollutants in the water. Vili Englund & Mikko Heino (1994) said that the perfect mussels for biomonitoring are the ones that are filtering during longer periods of time; they also stated that *U. tumidus* is more suitable for biomonitoring than *Anodonta anatine* because *U. tumidus* can ingest more food particles in the water.

Bivalves meet the requirements to be selected as a suitable monitoring organism. They are sedentary, abundant and available throughout the year. Furthermore, the organisms should have a manageable size and be hard enough to be handled in the laboratory (Kramer et al., 1989; Ismail et al., 2007).

1.4 Mussels characteristics (morphological and physiological) which determine their sensitivity to pollutions

The freshwater mussels have numerous features that differ from others. First, they have a soft body, which include the respiratory and digestive organs, together with other vital parts. The way they feed is by filtration: they filter large amount of water, including the matter dissolved in it. That makes them especially susceptible to toxic substances in the water, because mussels accumulate theses harmful substances in the gills and mantle cavity. The second one is a muscular foot, which extends from the two valves to help the mussel move, search and position in the river bottom. Studies suggest that younger mussels tend to go deeper in the sediment (Jonsson Annie *et al*., 2013) than older ones, which usually stay on the riverbed (surface) and are fairly static.

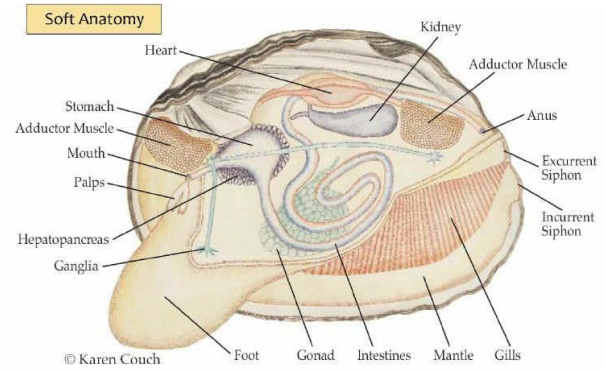


Figure 1. This simplified illustration shows the arrangement of the soft tissue body parts of a freshwater

Finally, the third characteristic is that freshwater mussels have two valves, one of the most important characteristics of all bivalves: it is what defines them, and there is an extraordinary variety in the shell form. In the case of *U. tumidus,* the shell is separated into two pieces or valves that connect at one of their ends by a ligament. The adductor muscles located in that area are the ones in charge of closing and opening the two valves of the shell. It has an elliptical shape and both shells are almost symmetric.

Figure 2. Both pictures of *Unio tumidus* in natural state

Mark Young (2005) in his research concludes that young mussels are more sensitive than adults in poor water conditions. He also stated that well-fed mussels are less sensitive than starving mussels and it is likely that other physiological states, such as gravidity affects the sensitivity as well. Another observation the author made is that adult mussels reduce toxin intake in the short term by closing their valves. For the sake of this study, however, it has discovered that the longer a mussel keeps their valves open, the better bioindicator it will be.

2. Aim

2.1 Testing of the suitability of *Unio tumidus* for the monitoring of water quality

The purpose of this study is to examine the ability of *Unio tumidus* to adapt to a biomonitoring system. The idea was to create an early warning system against sudden pollution of the water in order to give an immediate response or alarm. It was worked to identify the sub lethal toxicity of metals in mussels: *Unio tumidus,* their behaviour and how this system could be recorded on a computer.

For this, it is necessary to have a control over them and the movement of their shells, as it has been shown that the mussel reacts under stress conditions by automatically closing the shells. What it was did was to connect the mussels to the computer by using a sensor which measures the shell openness, with the help of a magnet. Their movement is registered showing a graphic on the screen. By analysing the obtained results, it could determine how mussels react after being exposed to different substances, and observe the maximum (full opening) and the minimum (closure) values.

We have to take into account species that are:

- Quite resistant to toxic substances
- Able to withstand the levels of toxicity limit for humans or other living beings.
- Mostly with an open shell
- Does not require much care

2.2 Analysis of the response of mussels to the presence of nitrate, ammonium nitrate and iron in water

The Maltański Reservoir, also known as the Malta Lake, situated in mid-western Poland, near the center of the City of Poznań was built in 1952 by damming the River Cybina. It is a shallow (mean depth of 3.1 m, maximum depth of 5.0 m) and small (area 64 ha) (Gramowska et al. 2010). In the summer season, it is intensively used for national and international canoeing and rowing competitions and serves as a bathing area for city residents.

This lake is classified as polymictic: there is one layer all the year and that the water mixes several times a year (Joniak et al. 2000). The Cybina river, whose catchment area is dominated by cultivated fields, flows into the lake and supplies it with nutrient-rich water with a prevalence of nitrates and phosphates that stimulates the primary production of phytoplankton in the Maltański Reservoir (Kozak & Gołdyn, 2004; Gołdyn & Szeląg-Wasielewska, 2005). In this research, it has tested mussels with two different concentrations of nitrate and ammonium nitrate to see how it affects them.

The reservoir is located near the city center where there are heavy traffic roads and residential areas. The lake's flow character has a strong impact on the water quality. Several studies corroborate, after analyzing mussels' soft tissue, that it has high concentration of heavy metals. Rzymski (2013) said that metal concentration in bivalves generally followed the level of contamination of their immediate environment. All the studied metals were detected in the soft tissues of three bivalve species, among them *U. tumidus*, collected from the Maltański Reservoir. The overall mean concentrations of metals decreased in the following order: Fe>Mn>Zn>Cu>Cr>Ni>Pb>Co>Cd (Rzymski et al. 2013). For that reason, it has tested them with two differents concentrations of iron in water.

3. Methodology

The present study was performed at the laboratory of Department of Ecology and Environmental Protection in Poznan University of Life Science during the period from March to June, 2016. The following paragraphs describe how we carried out the experiment with mollusc as biomonitors against water pollution.

3.1 Description of the biomonitoring system

A short and simple scheme can help us to better understand the purpose of this study. In Figure 3, one can see how the water we want to test is connected to a water treatment plant that is supplied to the residential area. On the other hand, there is another connection to a biomonitoring system: when the computer recognizes a pattern of abnormal behaviour by the presence of toxic substances, it automatically activates both the alarm and emergency system.

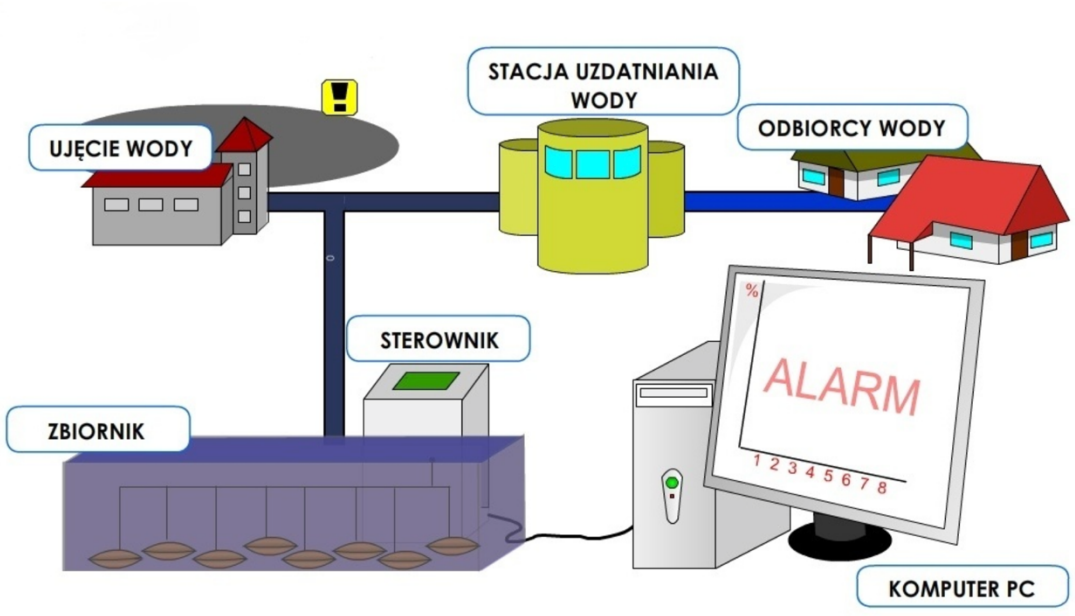


Figure 3. Measuring device set-up. water intake (ujęcie wody); water treatment station (stacja uzdatniania wody); inlet water (odbiorcy wody); computer pc (komputer pc); controller (sterownik); tank (zbiornik) Source: http://duszpasterstwo-wodkan-ochrsrod.pl/2012/07/18/robota-dla-mieczakow/

3.1.1 Type of tank and materials

To perform this experiment, it was used a glass tank. This is a very suitable material because it prevents the adhesion of particles to any surface, making the tank cleaning and maintenance tasks easier for the researchers. Whenever it was performed a toxicity test, the mussels were removed and it was used a filter brush to clean the tank walls. Later, with the aid of a hose it was emptied the tank water. It is noteworthy to mention that no type of detergent was used in this procedure since soaps or chemicals are difficult to remove later on, and they can cause a potential risk of contamination in the mussels' environment, which would eventually affect our results.

The tank has a volume of 100 dm^3, and we filled 60 dm^3 with tap water. Then, it was introduced the mussels that were already set up with a magnet, support, sensor and a granite stone to prevent the stand from moving. Finally, the sensor is connected to a data processor and, after performing a calibration process that will be later explained, we set up the computer in which the opening/ closing movement of the shells of eight mussels in real time will be registered (Figure 4).

The water in the tank is static. It mixes itself through a water impeller located on one side (Figure 5). What is intended with this process is that the irrigator helps distribute the substance as homogeneously as possible, and the concentration of toxicity remains constant for the mussels to have the reaction with the same concentration all the time.

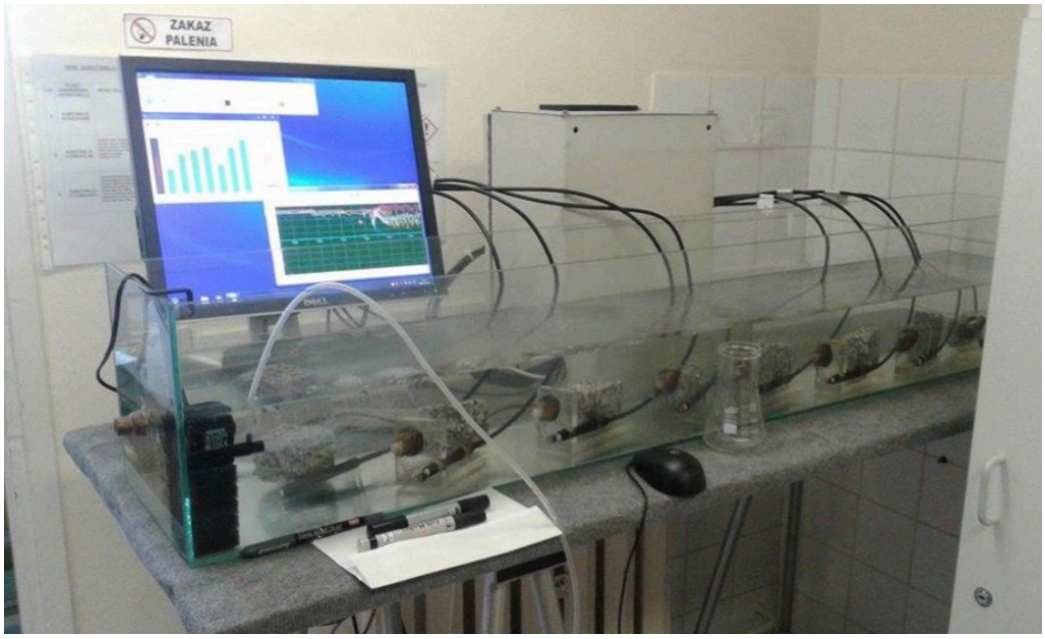


Figure 4. Picture taken in the laboratory of the faculty in which we can identify the final set-up used for the present research

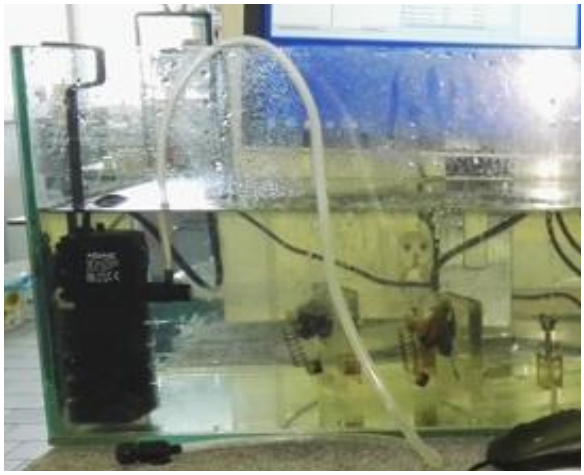


Figure 5. Pump impulsion. The substances are dosed by the pump that we installed in the system. This solution allows to run a non- invasive introduction of testing substances.

3.1.2 Preparing the mussels to research (quarantine period and its significance, the age of mussels, the number of individuals in the experiment)

The specimens used *Unio tumidus* were collected by hand in Malta lake from a maximum of 2 m deep. The selected mussels, between 45-50 mm shell lengths, were transported to the laboratory in a 2L sealed box filled with lake water. It has proceeded to clean them, to later place them in an aquarium with tap water at 18ºC temperature and gravel substrate (image 3). They were fed with food for aquarium fish once a week and they were fed until they were used for the experiment: mussels are able to fast for long periods of time (maximum three months). Later, mussels were put in quarantine for a month, allowing us to reject weak individuals.

Figure 6. Photography of the quarantine aquarium, there are free mussels and mussels stuck onto the socle to be installed later in the research system

After this time, the eight strongest molluscs were selected. Adults' specimen of *Unio Tumidus* were sampled in the tank. In the laboratory, the mussels were cleaned and individually prepared to measure their valve movement by fixing one of their valves to small glass support while the other valve remained free.

It was used a support made of methacrylate that will stick to the mussel's shell. It has been designed specifically for this experiment. The support's building process is described below:

1) Once the support is clean and wet, we applied silicone on the top left corner, so the silicone can be removed easily; this must be done every time you start a new test.

2) After the quarantine, the mussels were taken one by one and we tested if they were healthy by creating stimuli and see if they responded properly: by closing the valves.
3) The next steps is to apply silicone on the shells of those mussels that were healthier, as it is shown in the picture below (Figure 7).
4) After 24 hours, once the silicone is dry, we attach a metal spring to the mussels' shell that is also linked to magnet. It is noteworthy to mention that the magnet must be placed at right angles in the shell and that is cannot be too inclined (Figure 8). The reason is that the fixed element has to overlap completely the opening intended for the probe.
5) Finally, we waited for glue to dry and we placed the mussels with the sensor system into the water tank. The, it has repeated the procedure for all eight individuals.

Figure 7. Preparing the socle still wet with the silicon to steck the mussel.

Figure 8. Visual explanation of the mussel placement above the support. The two images framed in red are misplacement and picture framed in green it is correctly oriented on the support.

3.1.3 Calibration system

To perform an accurate calibration, it must perform the following steps for installing the system biomonitoring mussels:

1) Each mussel has its own channel for the sensor: the software has a transition mode called “CALIBRATION” that it has used for channel no. 1: we choose the operated arrows, place an operator console, push the ACK button for a second and finally push F button at the same time.
2) Then, with the probe into the water and without a magnet contact - to accept, push ACK after data stabilization.
3) Now, pull out the probe with the magnet; with a measurement of 20 mm removed from probe – the data displayed on console have to be stable and be about 225. To accept, push the ACK. At this stage, it is very important that the mollusc shells are closed.
4) The measurement with magnet (5 mm removed from probe) – the data displayed on console have to be stable and about 2500: the probe cannot be into the water; mussels’ shells must remain close. Once the data is stable, tighten the screw on the probe and put it into the water. You can now touch the mollusc, making sure the data is stable. After that, push the ACK to accept changes. Push ACK again to end the process.
5) Repeat this process with the other mussels.

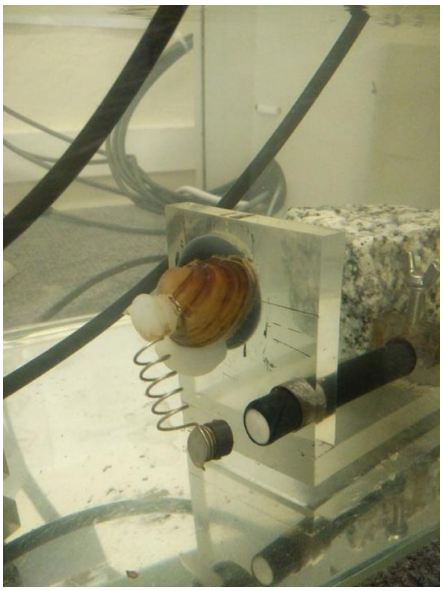


Figure 9. Mussels already installed and ready for biomonitoring.

There are several factors that can affect the results of the experiment, as misplacement of mussels' support or magnet. Thus, a bad sensor calibration can significantly affect the results.

After this process, the server can be activated. After calibration and activate the system, the user has to log on. The system is ready to use.

The system allows to control different parameters and provide further Information about the system. As follows, the different options that the user can visualize on the interface that is represented in Figure 10,

1) Reads the windows settings saved before,
2) Save the current windows settings,
3) Turn off the alarm,
4) Block the main window during the alarm,
5) Open the window of export events,
6) Open the window of export measures,
7) Hide the taskbar of function button,
8) Hide/ show the taskbar of current molluscs state,
9) Hide/ show the taskbar of current incident,
10) Show the window of current molluscs activity,
11) Show the window of the graph with current molluscs activity,
12) Show the window of incidents lists,
13) Show the window of settings (ONLY FOR SERVICEMAN),
14) Interrupt the current connections and show the window of server choosing,
15) Show the window of choosing the data to browse off-line,
16) Turn on/off the voice controlled (If is available),
17) Show the window with information about PROTE,
18) Show the window with information about program,
19) The setting of window transparency.

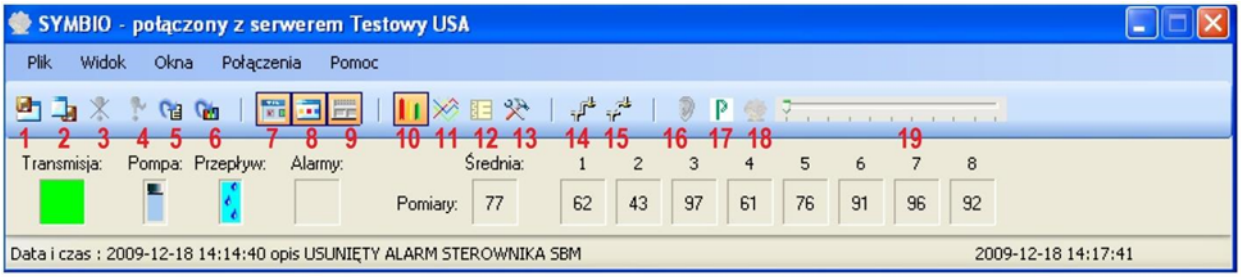


Figure 10. The taskbar of the main window of the system.

3.1.4 System errors

Visual examples of what happens when the process is not correctly done, as previously explained, can be seen the three images that follow. Poor calibration results will not represent reality and, therefore, they will not be conclusive. In addition to bad calibration, there are other factors that can directly interfere in the results, such as water quality and temperature among others. Mussels react differently to changes in the environment; for this reason, researchers need to very accurately follow the procedures in order to prevent that any other external factors (other than the substances that are the subject of study) alter the mussels' reaction, affecting the experiment results.

The following images, a screenshot of the Symbio software, shows the movement of the eight mussels. In the x-axis indicates the time and the y-axis shows the percentage of opening shells. The next image shows a bad calibration and it can be noticed how the mussels were suddenly closed between 10-40% (Figure 11). This is not accurate since a 0% ratio is expected: the sensor itself is represented with an aperture of 0% (i.e., when valves are fully closed) and a 100% aperture (i.e., fully opened); otherwise, the graph will not provide a reliable representation of the shells behavior. We know that the calibration process is inaccurate when a few mollusc are open just to a 70% or closed to a 40%; when mussels are resting, the percentage should be between 0 to 10% or between 80-100% when they are active.

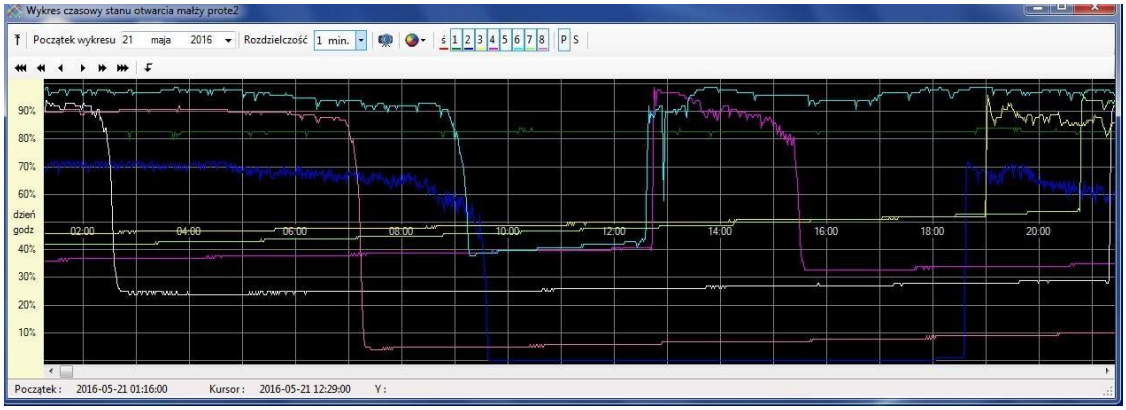


Figure 11. Bad calibration.

The following image represents a good calibration (Figure 12). One can observe how the starting point for all mussels is an opening of 0% and they open up over time. In this case, we made sure that when we measured the distance between the sensor and the magnet, the shells were completely closed so the program could take the correct reference. After an hour, we can see that the mussles are open between 80-90%.

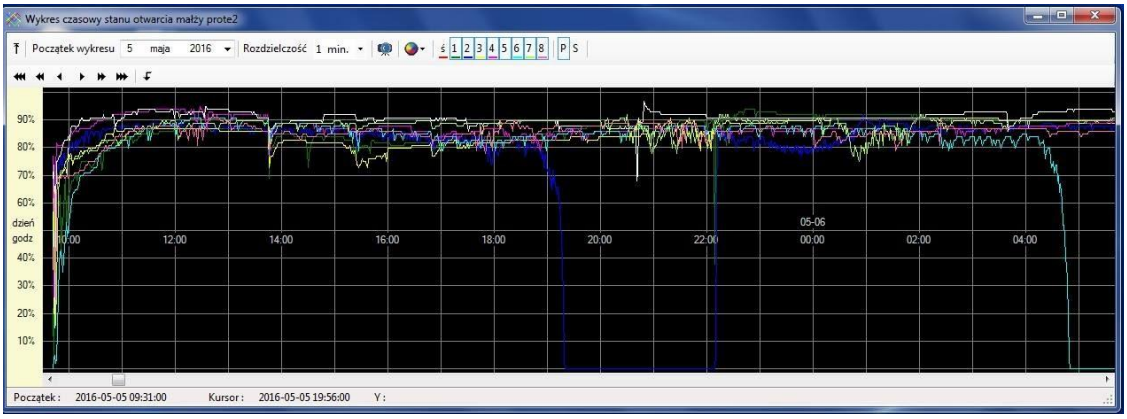


Figure 12. Good calibration

The result of a good calibration is shown in the picture below (Figure 13): all mussels opening ratio's are between 80% and 90% in the active state and between 0% and 10% when they are at rest. This would be the normal behavior of a mussel.

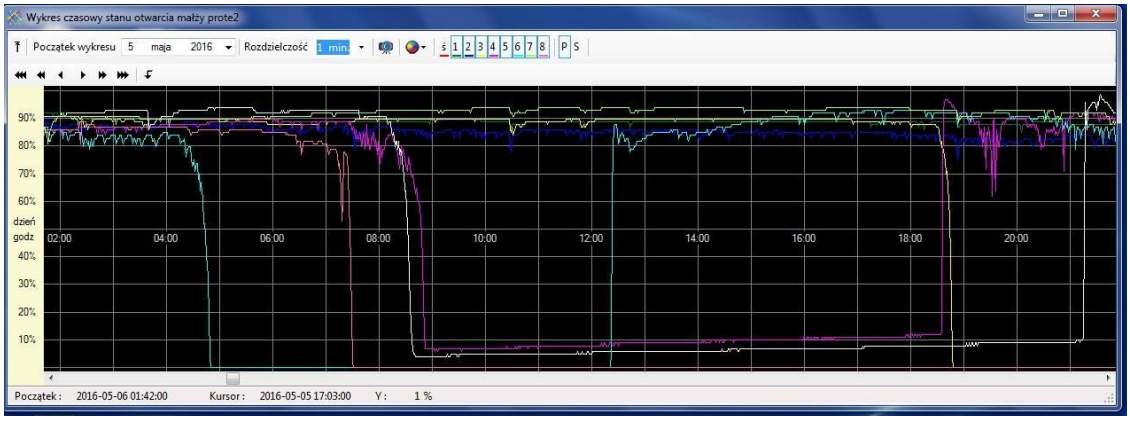


Figure 13. Result of good calibration

On the other hand, a good calibration is as important as keeping a good water quality for the mussels. They are so sensitive that poor water quality could interfere with the results of the research (Figure 14).

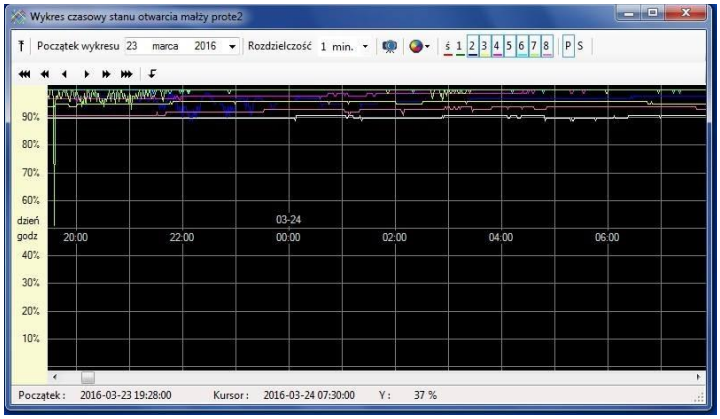


Figure 14. Good water quality

If the mussels' environment is not ideal, in the end one will not know if their behavior is due to water chemistry or the substance that we added as the objective of the research. In the following Figure 15, one can observe mussels being restless, in constant motion; most of the time, in a normal state, mussels would remain open, in a stable manner, filtering water to feed themselves and there would be moments when they will close their shells to rest. Thus, what it has observed in this picture is that mussels are not comfortable in the environment in which they are.

Figure 15. Bad water quality

It also noted that the temperature is a sensitive parameter for mussels and can alter the movement. Figure 16 shows drastic changes in mussels' behavior. As it has seen previously this is not within normal mussel' behavior.

Figure 16. Bad water temperature

That is why there are many factors to consider. Being so susceptible to any change in the parameters of the water, this alarm system should recognize behavior under toxic conditions; this would require to create identifying behavior patterns.

3.2 Description of the analysed substances

3.2.1 Dosing of substances

The experimental part of this study consisted of the following steps:

1) The first thing we did was filling the tank with clean tap water to 60 dm^3. We then prepared the mussels and their support device, and we wait for one day until the glue dries completely.
2) The next day we introduced the mussels into the tank and they were left there for 4 days in order for them to acclimate: if we started to record their movements from the first day, we could have errors in the results because it would take into account a period of adaptation to the new environment and we could have abnormal behavior.
3) On day 6 we prepared and added the substance into the tank with the help of a metering pump (Figure 5), so that the tested substance distributed as homogeneously as possible in the water: this way, all individuals received the same amount.
4) The results were collected 24 hours after adding the substance. Once we obtain the expected graphs, we proceeded to remove the system and clean the tank for the next experimental test.

The experiment has been done in accordance with the following algorithm:

Table 1. Experimental routine during seven days for each substance.

Day 1	Day 2	Day 3/4/5	Day 6	Day 7
Fill the tank with tap water	System calibration	Acclimatization	Substance's dosage	Collect the results
Preparing mussels				Remove the mussels
				Clean the tank

3.2.2 Analysis of the results

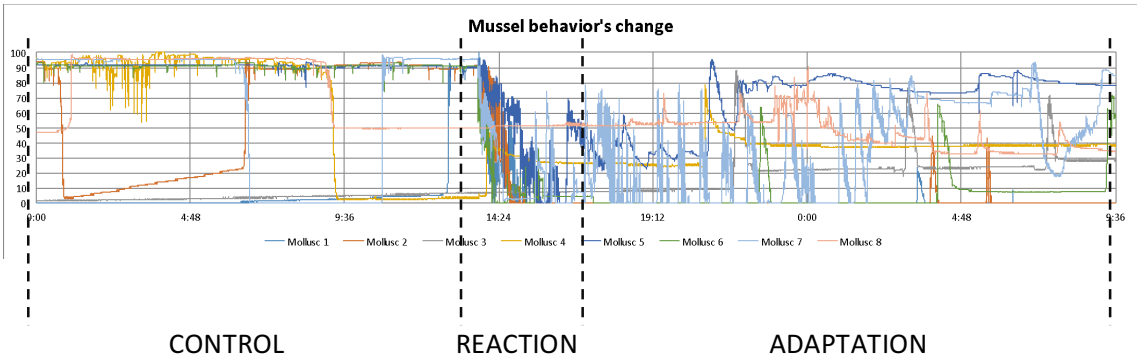
A total of 40 individuals were monitored for the behaviour with a differents substances. Before administering any treatment, we checked the mussels' normal behavioural status by recording them for a few hours (pre-exposure). After this pre-test, only those individuals that showed fully normal activity were kept for the toxicity test.

Visual observations show that the behavior of mussel may vary depending on change in water quality or other external factors such as temperature (seen in preceding graphs).

In regard to the changes in the behavioural waves between normal and stressful activities are shown in Figure 17.

In addition, an interpretation was made on the activity of mussels under experimentation, dividing their behavior in 3 states: (1) **control**: normal behavior without external disturbances, (2) **reaction**: right after the addition of the substance followed by an intense activity and (3) **adaptation**: mussels' behaviour is more stable for different reasons: the mussel tries to adapt to the new environment, it is weakened, or is dying.

A.



B.

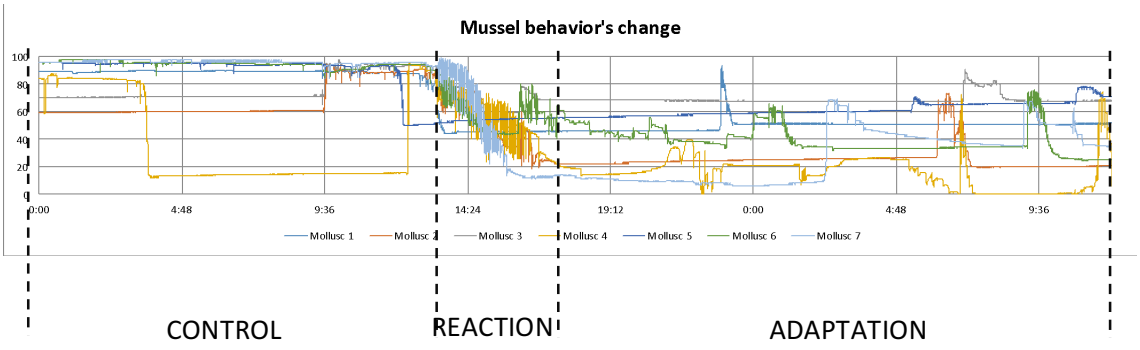


Figure 17. This graphic shows the mussles' behavior's change collecting all datas of more than one day of ammonium nitrate with 10mg l^{-1} (A) and 25mg l^{-1} (B)

From 0.00 a.m to 13.40 p.m we find mussels' normal behavioural activity: most of them are open for the longest time. However, a few of them are closed for several hours. Generally, mussels in

normal conditions are open, and this aperture ratio can be observed in the graphic from 80% to 100%. Before that time, the behavioural activity changes drastically, due to the substance that we added in their medium

The culture medium added for the tests was 0'2 mg L^{-1} and 1 mg L^{-1} of Fe^{+3}, 10 mg L^{-1} and 25 mg L^{-1} of NH_4NO_3 and 10 mg L^{-1} of $NO^-_{3(aq)}$.

For analysis of the results, on the one hand I have observed mussels' reaction to each substance. In the case of NH_4NO_3 and $NO^-_{3(aq)}$, I collected data from the first 3 hours after the substance is added, until the mussels tries to adapt to the new environment, considerably reducing its activity (adaptation state). These values were not interesting for two reasons: (1) the set of values of reaction time (immediately after introducing the substance) are very different from set of values obtained from the time of adaptation. Therefore, one cannot perform an analysis of data together because that would result in different interpretations; (2) we want a biomonitoring system that detects as soon as possible atypical behavior of mussels to activate the alarm. Thus, our goal is to collect the minimum set of values that are significant enough for a correct interpretation. In the case of iron (more on this later) however, we chose the values of 24 hours because the reaction of individuals was practically nil.

A graph showing the average values of the shells' openness taken every 15 minutes was created. This is a clear way to represent the overall mussel's activity.

Finally, I have analysed how many times shells' position was between 0% and 10% (Closed), 11% and 79% (Middle) and 80% and 90% (Open) for each mussel. This fluctuation was reflected in a histogram; it has calculated the standard deviation of the set values to obtain information about dispersion, which would result in a higher or lower mussels' activity.

4. RESULTS

4.1 Analysis of the response to the added substances

The mussels remain open most of the time, so it has to get a chart like the one you see below (Figure 18), which will be the representative of a control behavior, except for mussels 1 and 2, which had to be replaced by healthy mussels.

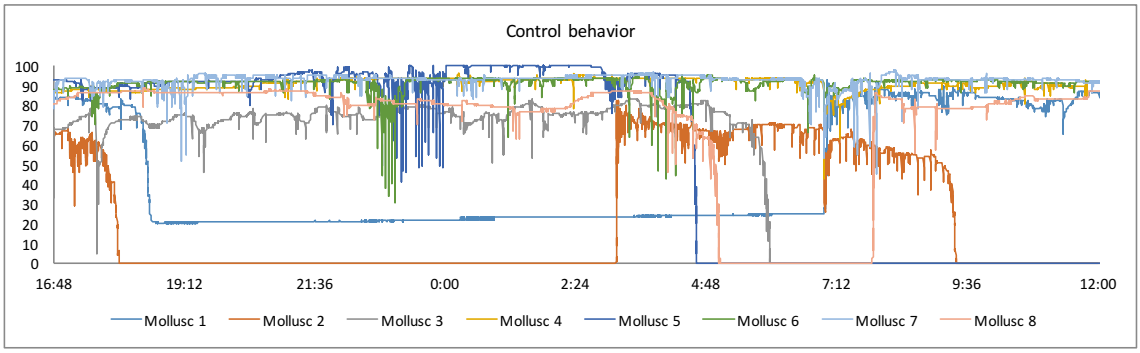


Figure 18. Control behavior of the eight mussles during 24 hours

When plotting graphs in Figure 19 and Figure 20 with the data collected from the mussels with the addition of iron, we note that the reaction is very slow regardless of the added concentrations. They have not the established control behavior, but they are kept open for a long time, so they are quite resistant to iron. One could say that with 0.2 mg / l is less toxic to mussels than 1 mg / l of iron in the middle, but still there is very little difference between the two graphs.

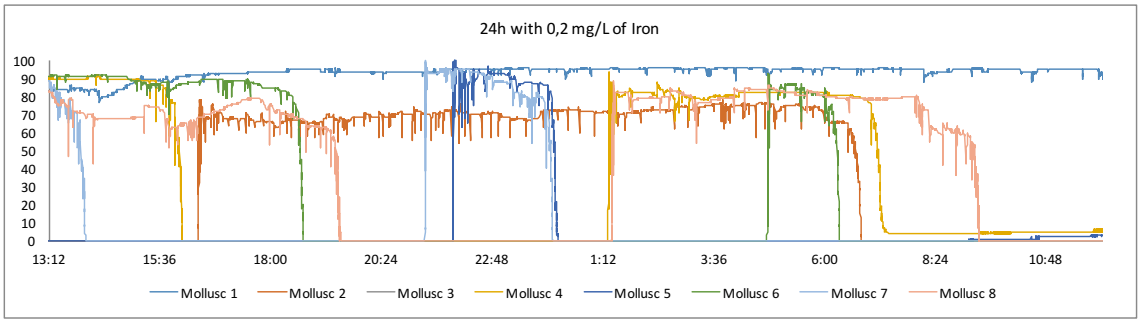


Figure 19. Behavior of the mussels with 0,2 mg/l of Fe^{+3} during 24 hours.

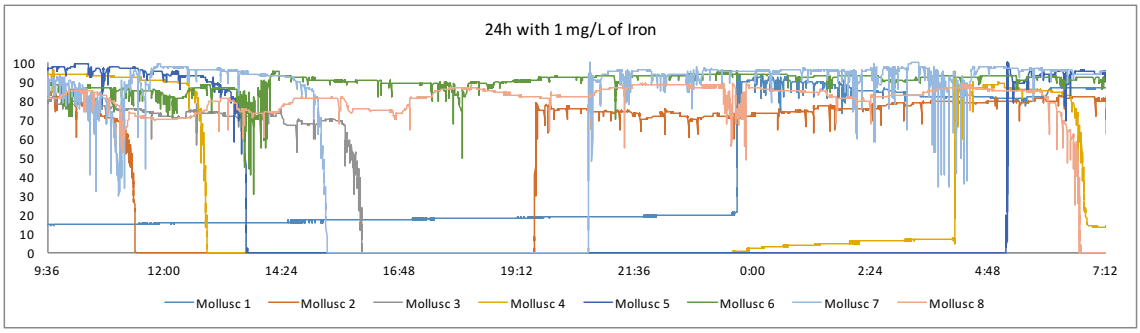


Figure 20. Behavior of the mussels with 1 mg/l of Fe^{+3} during 24 hours.

However, in the case of ammonium nitrate, the reaction is more drastic for the mussels, because such concentrations of ammonium nitrate are toxic to them. If we compare both concentrations of ammonium nitrate, mussels in

Figure 22 are most affected, significantly reducing its activity. This is opposed to what is seen

Figure 21, where mussels show an attitude of adaptation to new conditions. Hence, mussels 2, 3 and 4 in

Figure 21 and mussels 1 and 5 in

Figure 22 would not be representative.

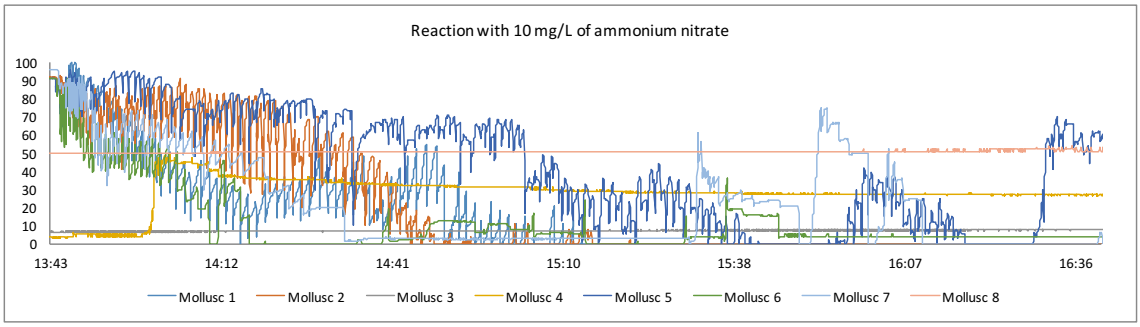


Figure 21. Behavior of the mussels with 10 mg/l of NH_4NO_3 during three hours.

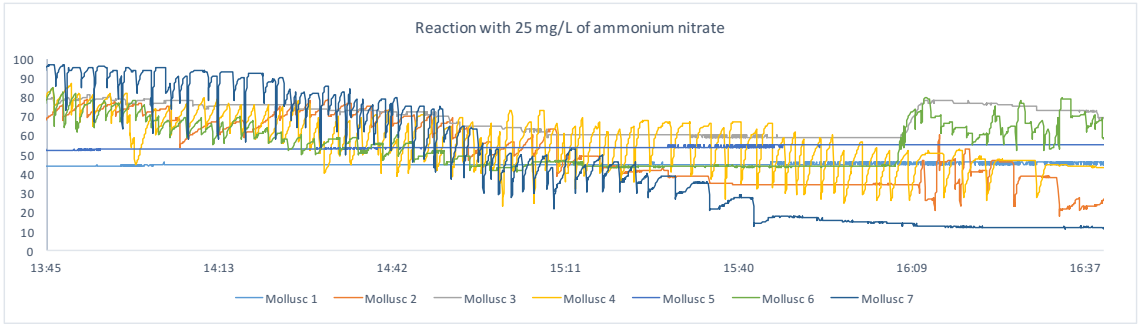


Figure 22. Behavior of the mussels with 25 mg/l of NH_4NO_3 during three hours

With regards to the behavior shown in the following chart (

Figure 23), we can see that mussels follow a similar behavior among them. They open quickly and try to stay open for about two hours, but at a certain point they close suddenly. When comparing the ammonium nitrate reactions, one possible interpretation is that nitrate are probably more tolerated than those exposed the ammonium nitrate.

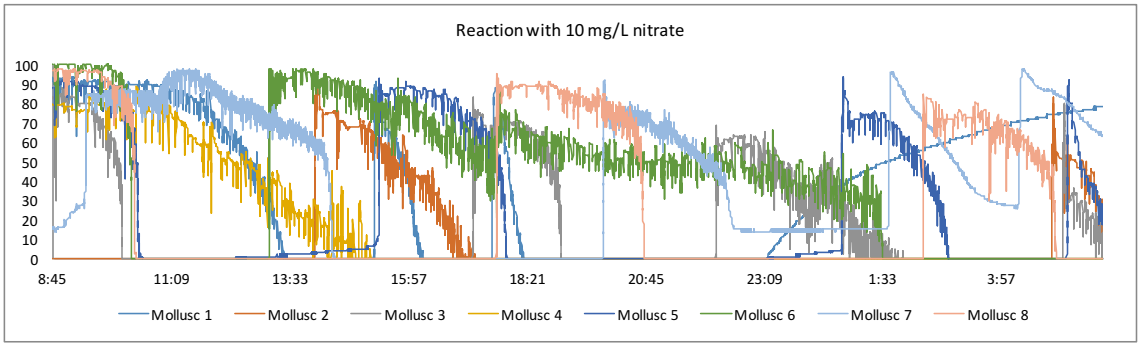


Figure 23. Behavior of the mussels with 10 mg/l of $NO^{-}_{3(aq)}$ during three hours

4.2 Analysis of the variation in response to various substances

A way to understand the reaction of the 8 mussels' altogether is shown in the following charts. Next graph is the control during 24 hours, it indicates how a normal state line should look like. Smooth fluctuations are observed and most of the time they remain open (Figure 24).

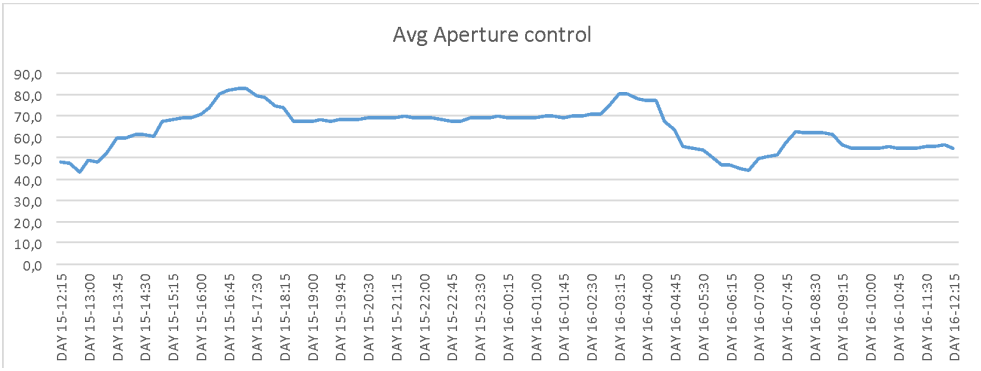


Figure 24. Graphical representation of the average percentage of opening every 15 minutes from the eight mussels without any substance

In Figure 25, with 0'2 mg/l of iron, the reaction of the change (indicate with red broken line) in the environment is not too notorious. We observe that the chart drops until a 15% average intermittently. Therefore, they react slowly with smooth drops and spikes to changes in the environment.

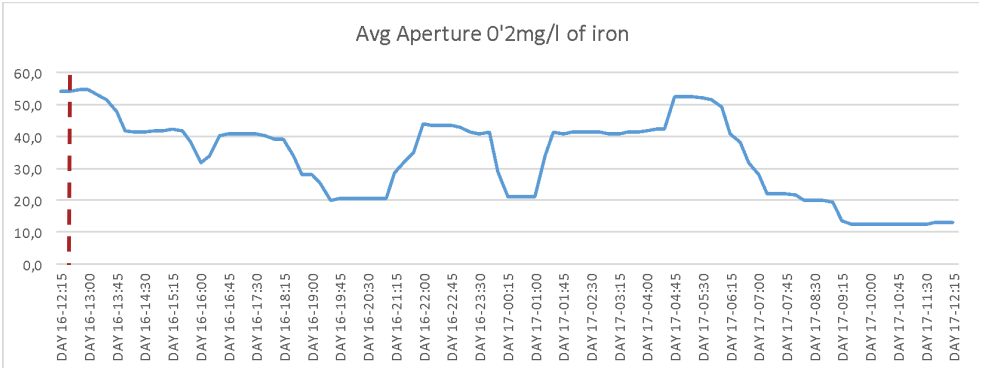


Figure 25. Graphical representation of the average percentage of opening every 15 minutes from the eight mussels with 0,2 mg/l of Fe

However, in the 1mg/l of iron (Figure 26), we could generally assume that the line is tending to do a slight concave curve, there are no fluctuations as seen previously. At first glance we can assume that they are more sensitive to 0.2 mg/l than with 1 mg/l of iron, but actually what is happening is the opposite, when they detect a higher quantity of iron the shell protects itself remaining close for a longer period of time. On the other hand, with less amount of iron the shell opens and closes more often.

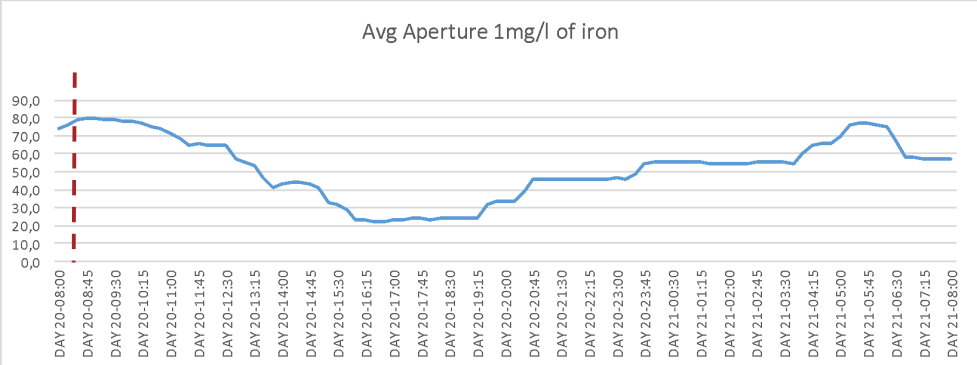


Figure 26. Graphical representation of the average percentage of opening every 15 minutes from the eight mussels with 1 mg/l of Fe^{+3}

We have obtained a different reaction in Figure 27, we added 10 mg/l of ammonium nitrate. In this case, we can clearly observe how the mussels change their relaxed behaviour (open shell) as they will be in their natural condition, to a stressed behaviour closing the shell quickly to protect themselves from the changes. Once the reaction time has past, we can see how the mussels try to adapt slowly to the environment, opening the shell slowly overtime, it is highly probable that this happens because the added concentration was not high enough to harm them.

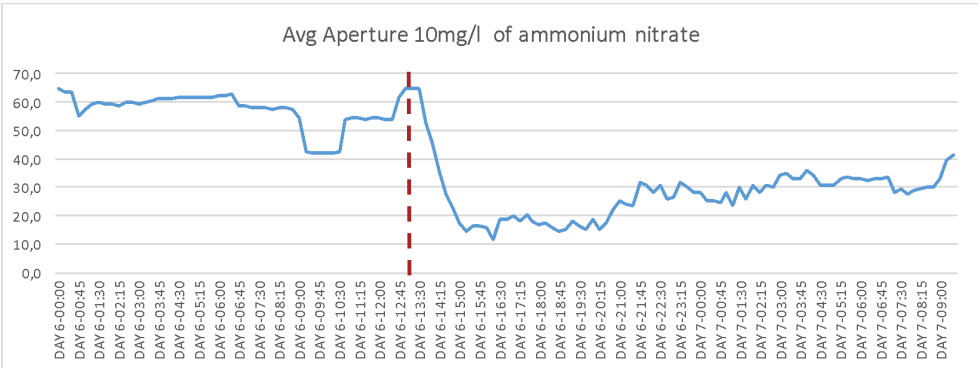


Figure 27. Graphical representation of the average percentage of opening every 15 minutes from the eight mussels with 10 mg/l of NH_4NO_3

When we add a higher concentration of ammonium nitrate the reaction of the mussels are to close themselves moderately. In Figure 28, descend significantly to a 40% remaining the same position as before. In this case, we could consider that this quantity of ammonium nitrate was harmful but it could have been lethal for the mussels.

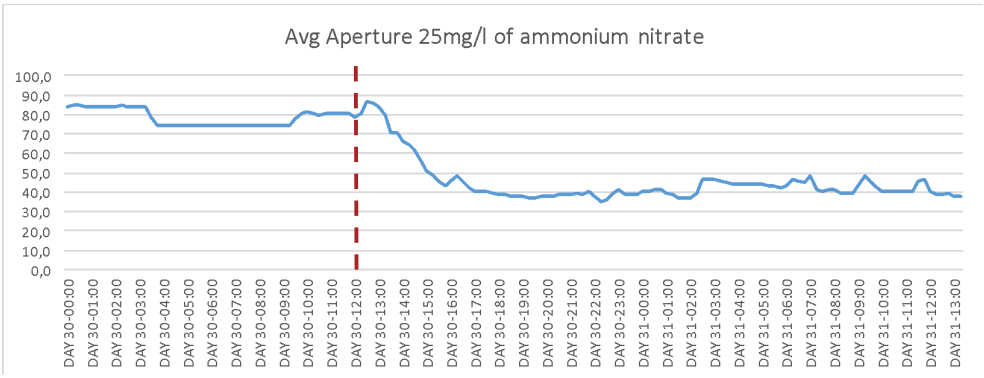


Figure 28. Graphical representation of the average percentage of opening every 15 minutes from the eight mussels with 25 mg/l of NH_4NO_3

The next figure, Figure 29 , shows a fluctuation very similar to Figure 25, with 10 mg/l of ammonium nitrate. Mussels react quickly to 10 mg/l nitrate, closing between 20% and 40%, keeping that apertura strip for the remaining time. The difference between both, is that in the former, the oscillations are slower than in the latter (Figure 25) which translates into sturdier individuals that are able to remain open for a longer time. Further, if we compare the previous two graphs, we can conclude that ammonium nitrate is more harmful to mussels than nitrate alone.

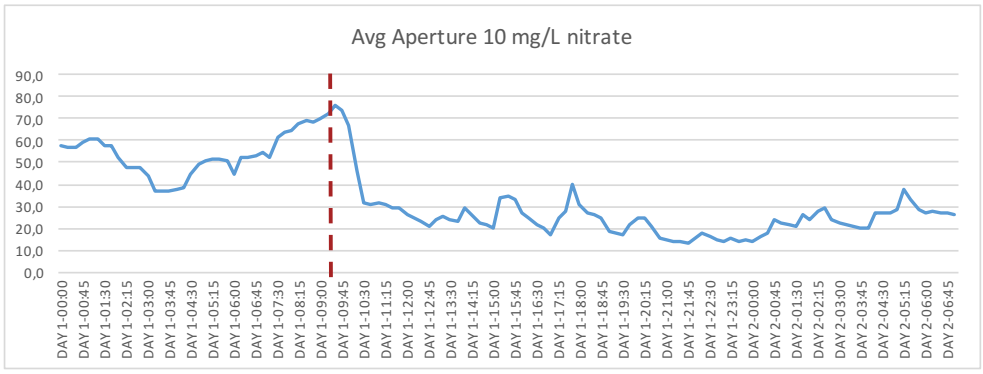


Figure 29. Graphical representation of the average percentage of opening every 15 minutes from the eight mussels with 10 mg/l of $NO^{-}_{3(aq)}$

4.3 Analysis of the variation in response of individual mussels

It is obvious that not all the specimens are going to behave the same way. In the following histograms we can observe them individually. On the axis-y (left side) is shown the time that they are in the same position in percentage and the right side is shown the standard deviation, and on the axis-x we have each specimen.

In Figure 30, shows the representation of the control one. Generally, it can see how the shells remain open with a high standard deviation, the collected interval values are fairly broad. For instance, the mollusc 5 has gone from 0 % to 100% (figure 31) aperture. However, the standard deviation of mollusc 4 has been lower due to its behaviour remaining stable during 90% of the time, only a 10% has been found in an intermediate position.

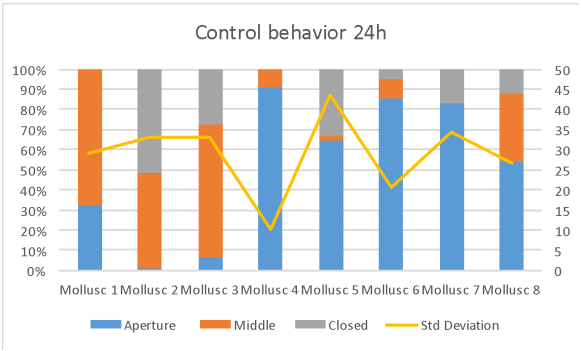


Figure 30. Representation in bars percentage of time spent in each position along with the standard deviation of the eight mussels without any substance

As seen in previous graphs, the reaction with iron has been slower. With the exception of mollusc 1, in Figure 31, all mussels remain close for a long time. In the case of mussel 3, that is closed all the time, it is possible that it was already weakened and maybe it was difficult for the mussel to get used to the new environment. On the other hand, Figure 32 shows how, with a 1 mg / l of iron, mussels react by closing the shells, except mussels 1 and 6. Thus, the use of a graph would necessary to understand their behaviour.

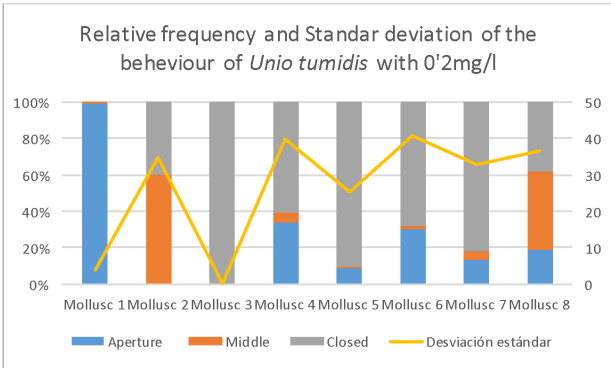


Figure 31. Representation in bars percentage of time spent in each position along with the standard deviation of the eight mussels with 0,2 mg/l of Fe^{+3}.

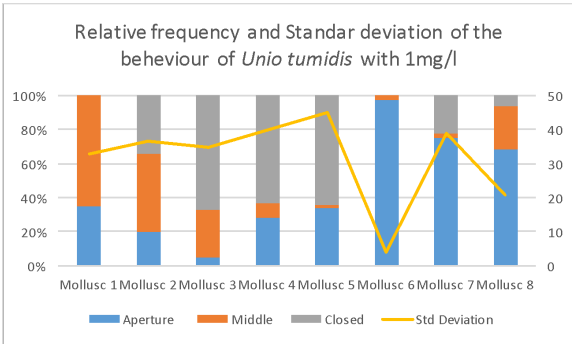


Figure 32. Representation in bars percentage of time spent in each position along with the standard deviation of the eight mussels with 1 mg/l of Fe^{+3}.

Comparing the two histograms, in both can be seen that mussels are closed, but at first glance it seems that with a lower concentration of iron, they are more susceptible. However, as in the previous section the same occurs: with a concentration of 0'2mg / L iron, the shells open between 80% and 100% and they closed suddenly when they noticed the added dose. Mussels tolerate less a higher dose of iron, and their movement is more gradual, slowly opening their shells.

In the case of iron, the analysis of the data is much more difficult because they have a very slow reaction. Quite the opposite occurs with ammonium nitrate, where histograms are more evident. In Figure 33, mussels stay longer closed and fail to reopen, only to the middle position. Mussel 3 was likely weak and could not bear the change in the cultivation medium to protect closing. The mussels 8 and 4 are in the middle position and with a standard deviation zero in the particular case of mussel 8, meaning that it was already dead. Also, in the case of mussels 4 with

a low standard deviation means that had a weak state of health as well. The three are not representative for the whole mussels' data sample.

In Figure 34 it is clear that a dose of 25mg / l is quite toxic to them. All mussels close to the middle position and with an average standard deviation of 12.2 points, low enough to consider that are not dead but impaired. In the case of mussels 1 and 5, the same as in the previous graph, they died and they are, therefore, not representative. Mussel 7 is the strongest one, and with the help of the graph, one can see how this mussel takes longer to react to the added dose.

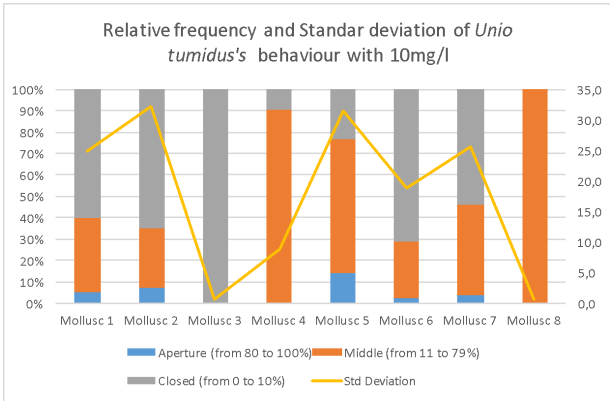


Figure 33. Representation in bars percentage of time spent in each position along with the standard deviation of the eight mussels with 10 mg/l of NH_4NO_3

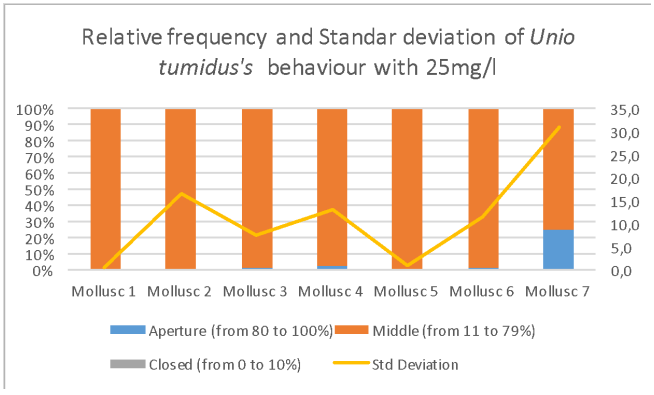


Figure 34. Representation in bars percentage of time spent in each position along with the standard deviation of the eight mussels with 25 mg/l of NH_4NO_3

As shown in the following histogram (Figure 35), most mussels are closed, so the muscle that

makes shells close is still alive, but with a very high standard deviation (i.e., have dramatic variations in the opening of shells). The analysis compared with figure 33 would show, once again, that ammonium nitrate is more harmful than the nitrate itself.

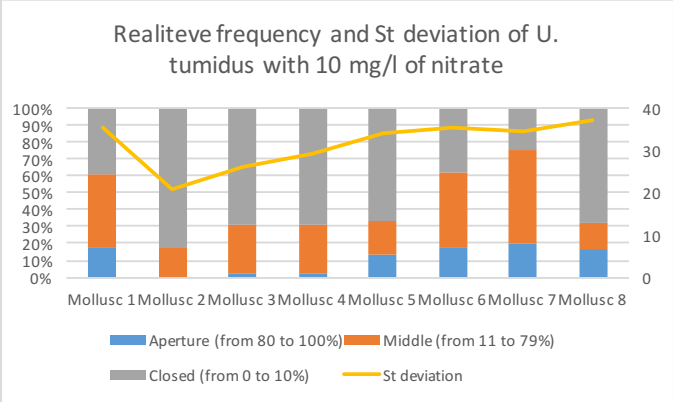


Figure 35. Representation in bars percentage of time spent in each position along with the standard deviation of the eight mussels with 10 mg/l of $NO^{-}_{3(aq)}$

5. Discussion

The way that the mussels reacted in front of iron, regardless of the dose added, has been much slower than with NH_4NO_3 and $NO^-_{3(aq)}$. The reaction rate of the mussels increased in the following order: 0'2 mg L^{-1} Fe^{+3} >> 1 mg L^{-1} Fe^{+3} > 10 mg L^{-1} of $NO^-_{3(aq)}$ > 10 mg L^{-1} NH_4NO_3 > 25 mg L^{-1} NH_4NO_3. It means that the mussels are more resistant to iron: this may be due to the ability to accumulate heavy metals. There is a study that show how the degree of metal accumulation in soft tissues followed the same order and water quality as in the associated sediments thereby indicating a strong relationship between ambient and biological concentrations (Rzymski et al. 2014). This is in general agreement with observations from other investigations on metal bioaccumulation involving unionids. As a result, it can say that mussels are more resistant to iron than to NH_4NO_3 and $NO^-_{3(aq)}$.

Furthermore, we observed some variations in behavior that were attributed to 'individual personalities': there were individuals that behaved differently to what was expected or commonly established. This reaction was related to a state of poor health and age, and also, to those mussels that were still at an intensive development stage and as a result had a high metabolic demand on essential elements that resulted in a high capacity to accumulate iron in their tissues. In order to solve this problem in future research, it has suggested two options: (1) use more than 8 mussels for the control and the treatment or (2) use an internal standard, which increases the number of mussels treated without increasing the experimental error. The latter option is the most preferable one. As a result, we have seen that the mussels have a similar reaction to the same substance and we can create patterns of behavior to identify each substance.

6. Conclusion

In the present study, we tested 40 individuals of Unio Tumidus to obtain the mussels' reaction for 0'2 mg L^{-1} and 1 mg L^{-1} of Fe^{+3}, 10 mg L^{-1} and 25 mg L^{-1} of NH_4NO_3 and 10 mg L^{-1} of $NO^-_{3(aq)}$. Mussels reacted differently to each substance at different concentrations.

We have observed that at low polluted concentrations, organisms can show resistance/tolerance or they may even attempt to adapt to the new conditions, since the reaction can be slower, or less obvious, or both (it depends on the pollution level). Furthermore, at higher concentrations the reaction is faster, in which case there is no possibility of resistance or adaptation to the new conditions.

Otherwise, we have seen that the mussels have a similar reaction to the same substance and we can create patterns of behavior to identify each substance.

There is few information about mussels' biomonitoring; further work needs to be undertaken to standardize these methods for acceptance as an early warning system. Hence this topic can be considered as an uncharted area, and further research is needed to deep dive in the mussels in particular, or mollusks in general, to understand their behaviour when they are exposed to different substances (as analysed in the present study) as well as to other substances that may be harmful for human health and can be found in polluted water.

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8. Appendix

Table 2. Descriptive statistic of control' behavior of the eight mussels

	Mollusc 1	*Mollusc 2*	*Mollusc 3*	*Mollusc 4*	*Mollusc 5*	*Mollusc 6*	*Mollusc 7*	*Mollusc 8*
Mean	46,3	31,7	53,3	88,1	61,9	84,6	77,6	71,3
St Error	0,1	0,1	0,1	0,0	0,2	0,1	0,1	0,1
Median	25	0	71	91	89	92	93	80
Mode	21	0	0	94	0	92	94	86
St Deviation	29,1	33,0	33,0	10,1	43,5	20,5	34,5	26,6
Sample Variance	848,2	1091,9	1086,8	102,2	1894,8	420,8	1192,8	710,0
Kurtosis	-1,7	-1,9	-1,0	18,8	-1,5	11,3	1,2	3,1
Skeweness	0,5	0,1	-1,0	-4,0	-0,7	-3,5	-1,8	-2,2
Range	76	84	84	67	100	95	100	89
Minimum	20	0	0	29	0	0	0	0
Maximum	96	84	84	96	100	95	100	89
Sum	3543795	2427387	4075466	6736237	4732763	6471330	5932333	5451144
Count	76460	76460	76460	76460	76460	76460	76460	76460
Aperture (from 80 to 100%)	32,5	1,3	7,0	90,8	64,9	85,3	82,8	54,6
Middle (from 11 to 79%)	67,5	47,6	65,8	9,2	2,4	9,9	0,8	33,6
Closed (from 0 to 10%)	0,0	51,1	27,2	0,0	32,7	4,9	16,4	11,8

Table 3. Descriptive statistic of the mussels' behavior with 0,2 mg l^{-1} Fe^{+3} in the water

	Mollusc 1	*Mollusc 2*	*Mollusc 3*	*Mollusc 4*	*Mollusc 5*	*Mollusc 6*	*Mollusc 7*	*Mollusc 8*
Mean	93,2	41,9	0,0	33,8	8,4	27,9	15,0	46,2
St Error	0,0	0,1	0,0	0,1	0,1	0,1	0,1	0,1
Median	95	67	0	5	0	0	0	68
Mode	95	0	0	0	0	0	0	0
St Deviation	3,9	34,6	0,0	40,0	25,6	40,6	32,7	36,7
Sample Variance	15,1	1198,6	0,0	1600,5	655,6	1646,7	1070,0	1344,7
Kurtosis	4,0	-1,8	0,0	-1,7	6,2	-1,4	1,2	-1,7
Skeweness	-2,2	-0,4	0,0	0,5	2,9	0,8	1,8	-0,4
Range	24	78	0	94	100	93	100	89
Minimum	72	0	0	0	0	0	0	0
Maximum	96	78	0	94	100	93	100	89
Sum	7149223	3211307	0	2591257	644504	2139284	1148021	3545786
Count	76721	76721	76721	76721	76721	76721	76721	76721
Aperture (from 80 to 100%)	99,4	0,0	0,0	33,8	8,8	30,1	13,4	19,1
Middle (from 11 to 79%)	0,6	59,6	0,0	5,8	0,5	2,3	4,3	43,2
Closed (from 0 to 10%)	0,0	40,4	100,0	60,3	90,7	67,6	82,3	37,7

Table 4. Descriptive statistic of the mussels' behavior with 1 mg l^{-1} Fe^{+3} in the water

	Mollusc 1	*Mollusc 2*	*Mollusc 3*	*Mollusc 4*	*Mollusc 5*	*Mollusc 6*	*Mollusc 7*	*Mollusc 8*
Mean	41,3	50,3	24,0	28,7	33,0	90,0	71,9	76,1
St Error	0,1	0,1	0,1	0,1	0,2	0,0	0,1	0,1
Median	19	73	0	4	0	91	94	82
Mode	16	0	0	0	0	92	0	85
St Deviation	32,8	36,4	34,8	39,9	44,8	3,9	39,1	20,6
Sample Variance	1078,0	1322,2	1208,9	1596,0	2004,6	14,9	1528,4	424,4
Kurtosis	-1,6	-1,6	-1,4	-1,2	-1,6	9,7	-0,4	8,8
Skeweness	0,6	-0,6	0,8	0,9	0,6	-2,3	-1,3	-3,2
Range	83	84	87	96	100	64	100	89
Minimum	12	0	0	0	0	31	0	0
Maximum	95	84	87	96	100	95	100	89
Sum	3165271	3856969	1842448	2195399	2531733	6897729	5508196	5832037
Count	76620	76620	76620	76620	76620	76620	76620	76620
Aperture (from 80 to 100%)	35,3	20,3	4,9	28,6	34,4	97,8	74,6	68,8
Middle (from 11 to 79%)	64,7	45,7	27,8	7,8	1,0	2,2	3,1	25,0
Closed (from 0 to 10%)	0,0	34,0	67,3	63,5	64,6	0,0	22,3	6,3

Table 5. Descriptive statistic of the mussels' behavior with 10 mg l^{-1} NH_4NO_3 in the water

	Mollusc 1	*Mollusc 2*	*Mollusc 3*	*Mollusc 4*	*Mollusc 5*	*Mollusc 6*	*Mollusc 7*	*Mollusc 8*
Mean	16,4	22,0	7,2	28,0	43,3	12,1	21,6	50,9
St Error	0,3	0,3	0,0	0,1	0,3	0,2	0,3	0,0
Median	0	0	7	28	48	4	3	51
Mode	0	0	7	27	0	4	3	51
St Deviation	24,9	32,1	0,6	8,9	31,5	18,8	25,6	0,5
Sample Variance	620,6	1030,5	0,3	80,0	989,3	352,4	655,7	0,3
Kurtosis	1,9	-0,8	-0,1	2,4	-1,5	5,2	0,0	1,5
Skeweness	1,6	1,0	0,1	-1,4	-0,1	2,3	1,1	0,0
Range	100	93	2	46	96	91	96	4
Minimum	0	0	6	3	0	0	0	50
Maximum	100	93	8	49	96	91	96	54
Sum	157358	210406	68637	267651	414656	115802	207116	48743
Count	9571	9571	9570	9571	9571	9571	9571	9571
Aperture (from 80 to 100%)	5,3	7,2	0,0	0,0	14,0	2,1	3,7	0,0
Middle (from 11 to 79%)	34,3	27,6	0,0	90,2	62,5	26,9	42,7	100,0
Closed (from 0 to 10%)	60,4	65,2	100,0	9,8	23,4	70,9	53,6	0,0

Table 6. Descriptive statistic of the mussels' behavior with 25 mg l^{-1} NH_4NO_3 in the water

	Mollusc 1	*Mollusc 2*	*Mollusc 3*	*Mollusc 4*	*Mollusc 5*	*Mollusc 6*	*Mollusc 7*
Mean	45,1	51,2	68,8	56,3	54,0	55,6	47,2
St Error	0,0	0,2	0,1	0,1	0,0	0,1	0,3
Median	45	48	72	56	54	54	41
Mode	45	34	60	47	54	44	12
St Deviation	0,5	16,7	7,6	13,0	0,9	11,5	31,1
Sample Variance	0,3	279,2	57,8	168,8	0,8	133,1	968,4
Kurtosis	0,6	-1,4	-1,6	-0,8	-0,8	-1,0	-1,5
Skeweness	0,2	0,1	-0,2	0,0	-0,4	0,5	0,3
Range	2	61	22	64	3	44	86
Minimum	44	18	59	23	52	41	11
Maximum	46	79	81	87	55	85	97
Sum	431683	489574	658961	538417	517113	532274	451733
Count	9571	9571	9571	9571	9571	9571	9571
Aperture (from 80 to 100%)	0,0	0,0	1,8	3,2	0,0	1,6	24,7
Middle (from 11 to 79%)	100,0	100,0	98,2	96,8	100,0	98,4	75,3
Closed (from 0 to 10%)	0,0	0,0	0,0	0,0	0,0	0,0	0,0

Table 7. Descriptive statistic of the mussels' behavior with 10 mg l^{-1} $NO^{-}_{3(aq)}$ in the water

	Mollusc 1	*Mollusc 2*	*Mollusc 3*	*Mollusc 4*	*Mollusc 5*	*Mollusc 6*	*Mollusc 7*	*Mollusc 8*
Mean	39,9	9,3	16,3	18,1	23,2	40,0	43,4	25,2
St Error	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1
Median	44	0	0	0	1	47	51	0
Mode	0	0	0	0	0	0	0	0
St Deviation	35,6	20,9	26,4	29,3	34,0	35,2	34,3	37,3
Sample Variance	1267,9	435,7	694,4	858,3	1157,5	1241,2	1179,2	1394,0
Kurtosis	-1,7	2,9	0,0	-0,3	-0,8	-1,4	-1,6	-1,1
Skeweness	0,1	2,1	1,3	1,2	1,0	0,1	0,0	0,9
Range	94	92	88	89	95	100	98	98
Minimum	0	0	0	0	0	0	0	0
Maximum	94	92	88	89	95	100	98	98
Sum	2854050	662948	1168779	1298847	1662537	2860970	3111109	1805561
Count	71610	71610	71610	71610	71610	71610	71610	71610
Aperture (from 80 to 100%)	17,4	0,4	2,7	2,8	13,7	17,5	20,1	16,8
Middle (from 11 to 79%)	43,9	17,7	28,7	28,1	19,6	44,6	55,5	15,6
Closed (from 0 to 10%)	38,6	81,9	68,6	69,1	66,6	37,9	24,4	67,6